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Research Article

qPCR validation and field application of a rapid RPA-LFD assay for monitoring the invasive alligator gar (*Atractosteus spatula*)

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Abstract

Understanding species' distributions beyond their native ranges is essential for monitoring non-native species occurrences. The alligator gar (*Atractosteus spatula*), native to North America and listed as a key invasive species in China's Catalogue of Key Managed Invasive Alien Species, poses significant threats to aquatic ecosystems. To enable rapid on-site detection, we developed and validated novel molecular assays based on recombinase polymerase amplification combined with lateral flow dipstick (RPA-LFD) and quantitative PCR (qPCR). We designed species-specific primers and probes, optimized reaction conditions, and evaluated assay performance. Both RPA-LFD and qPCR exhibited high sensitivity, with detection limits as low as 4 copies/μL, and strong specificity for *A. spatula* without cross-reactivity to non-target species. Under optimized conditions, the RPA-LFD assay achieved results within 15 minutes at 38 °C. Field testing in a natural breeding pond confirmed reliable detection, and the best-performing RPA assay (RPAS1) showed comparable efficiency to the qPCR gold standard. This study provides robust, field-deployable tools that empower early detection and proactive monitoring of invasive species, with strong potential for application in citizen science and large-scale biodiversity surveillance.

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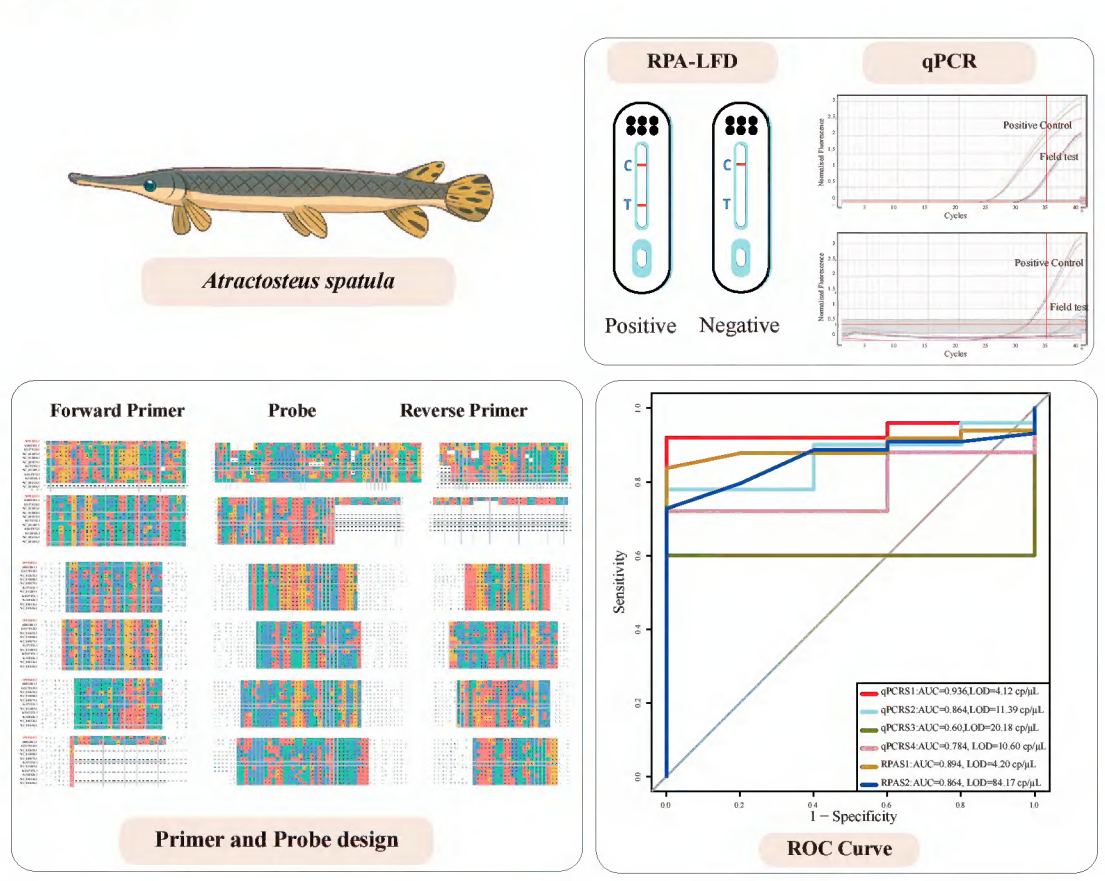
Graphic abstract



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Key words: Biosecurity, invasive species monitoring, qPCR, RPA-LFD

Introduction

Invasive species exert globally detrimental impacts on aquatic ecosystems through biodiversity compromise and economic disruption, with biological invasions recognized as the second greatest threat to global biodiversity after habitat alteration (Peller and Altermatt 2024). More than 39,215 alien species have been recorded worldwide; the effects of invasive non-native species can occur long after their initial introduction (Seebens et al. 2023). The alligator gar, *Atractosteus spatula* (Lepisosteidae, Lepisosteiformes), is native to North America and primarily inhabits the Mississippi River basin (Smith 1979; Collette et al. 2019). However, initially introduced through the ornamental trade, this apex predator has since been documented in numerous countries beyond its native range, including Singapore, India, and Indonesia, with established invasive populations worldwide (Collette et al. 2019; Manna et al. 2021; Xie et al. 2023). Designated as China's priority managed invasive alien species, the establishment of *A. spatula* has led to local extirpations of native fish populations in multiple Chinese provinces, including Guangdong, Henan, Yunnan, Hunan and Guangxi, and fundamentally restructuring trophic networks (Li and Zhang 2023; Xie et al. 2023). Furthermore, model projections indicate that large areas of southwest, south, central, and eastern China are suitable for the distribution of the *A. spatula* (Salnikov 2010; Liu et al. 2023). Therefore, early detection of this invasive species is essential to mitigate further ecological and economic damage.

The introduction and spread of aquatic invasive species (AIS) pose severe threats to aquatic ecosystems, underscoring the critical need for early detection (Thomas et al. 2020). Conventional monitoring approaches for invasive aquatic species, including visual census, net-based sampling, and morphological identification, face persistent challenges in accuracy and operational efficiency (Ogidi et al. 2024). Visual surveys frequently underestimate species presence in turbid or vegetated habitats, especially while morphological diagnostics prove unreliable in early life stage due to the lack of distinct individual characteristics (Beng and Corlett 2020). Furthermore, trap-based methods are time intensive and expensive, exacerbating ecological disturbance through non-target bycatch which hindering large-scale surveillance. Nowadays, genetics and genetic tools are playing an increasingly important role in providing information for the management of invasive species (Bohmann et al. 2014; Huang et al. 2022). Unlike morphology-based identification, which is often constrained by juvenile morphology or cryptic speciation, environmental DNA (eDNA) offers unambiguous genetic evidence that significantly enhances taxonomic resolution (Ruppert et al. 2019). This approach effectively overcomes the limitations associated with traditional morphological methods, providing complementary for detection for invasive species often being overlooked (Ruppert et al. 2019; Sahu et al. 2025). Its application in AIS detection has been demonstrated in diverse systems, including Bluegill sunfish in Japan (Takahara et al. 2013), Asian carps in the U.S. (Jerde et al. 2013), Rusty crayfish (Dougherty et al. 2016), and Round goby in North America (Balasingham et al. 2018).

Contemporary biosafety surveillance employs a range of methodologies, among which molecular diagnostic platforms have seen rapidly growing adoption in

invasion biology due to their sensitivity, specificity, and potential for field-based application (Rishan et al. 2023). The monitoring of invasive species based on eDNA commonly employs species-specific primers coupled with conventional PCR amplification, as demonstrated in studies of the golden mussel (*Limnoperna fortunei*) (Guo et al. 2024) and the invasive jellyfish (*Craspedacusta sowerbii*) (Blackman et al. 2022). Meanwhile, quantitative real-time polymerase chain reaction (qPCR) remains the gold standard for species-specific detection, offering high sensitivity and precise quantification through cycle threshold measurements (Feckler et al. 2017; Hernandez et al. 2020). In invasive species surveillance, qPCR-based eDNA assays have demonstrated superior detection efficacy and sensitivity compared to traditional morphological surveys, as evidenced by surveillance programs targeting the zebra mussels (*Dreissena polymorpha*), Red Swamp Crayfish (*Procambarus clarkii*), Sea trout (*Salmo trutta*), Red lionfish (*Pterois volitans*) and endangered Macquarie perch (*Macquaria australasica*) surveillance programs (Tréguier et al. 2014; Bylemans et al. 2017; Dahlquist et al. 2025; Kim et al. 2025; Viehl et al. 2025). However, the reliance on laboratory infrastructure and prolonged turnaround times limits its utility for rapid on-site decision-making. In contrast, isothermal nucleic acid amplification technique, recombinase polymerase amplification combined with lateral flow dipstick (RPA-LFD) demonstrates operational advantages through single-temperature reactions, rapid detection timelines (<20 minutes), and field-deployable workflows, while eliminating primer multiplicity requirements (Lobato and O'Sullivan 2018). The successful integration of RPA into various detection platforms has been demonstrated in species such as the Chinese Giant Salamander (*Andrias davidianus*) (Ling et al. 2024), the polychaete *Sabella spallanzanii* (Zirngibl et al. 2022), the common carp (*Cyprinus carpio*) and medaka (*Oryzias latipes*) (Yang et al. 2024), highlighting its versatility and practicality for field applications.

Although eDNA techniques are increasingly applied in invasive species monitoring, a rapid, field-deployable detection method for *A. spatula* is still lacking. Thus, the objectives of this study were to: i) develop the first RPA-LFD assay for the detection of *A. spatula*; ii) optimize and evaluate its performance against the qPCR gold standard; and iii) verify its reliability through field experiments and demonstrate its potential for application in citizen science–driven large-scale biosafety monitoring.

Material and methods

Design and screen of primers and probes

Mitochondrial genomes (mitogenomes) sequences of the *A. spatula* and the 11 comparative species were retrieved from NCBI database with accession numbers: *Acipenser sinensis* (KJ174513.1; Liao et al. 2016), *A. spatula* (AP004355.1; Inoue et al. 2003), *Carassius auratus* (NC_006136.1; Guo et al. 2007), *Coilia nasus* (NC_009579.1; Lavoué et al. 2007), *Cyprinus carpio* (NC_001606.1; Chang et al. 1994), *Hemiculter leucisculus* (KF647872.1; Dong et al. 2015), *Lepisosteus oculatus* (AB042861.1; Inoue et al. 2003), *Misgurnus anguillicaudatus* (NC_011209.1; He et al. 2008), *Nile tilapia* (GU370125.1; He et al. 2011), *Pseudorasbora parva* (KJ135626.1; Xu et al. 2016), *Rhinogobius giurinus* (NC_022692.1; Liang et al. 2012) and *Tachysurus fulvidraco* (NC_015888.1; Xie et al. 2015) (Fig. 1). Candidate genomic regions were identified through multiple sequence alignment using MEGA 11 (Tamura et al. 2021), prioritizing loci with <5% intraspecific variation

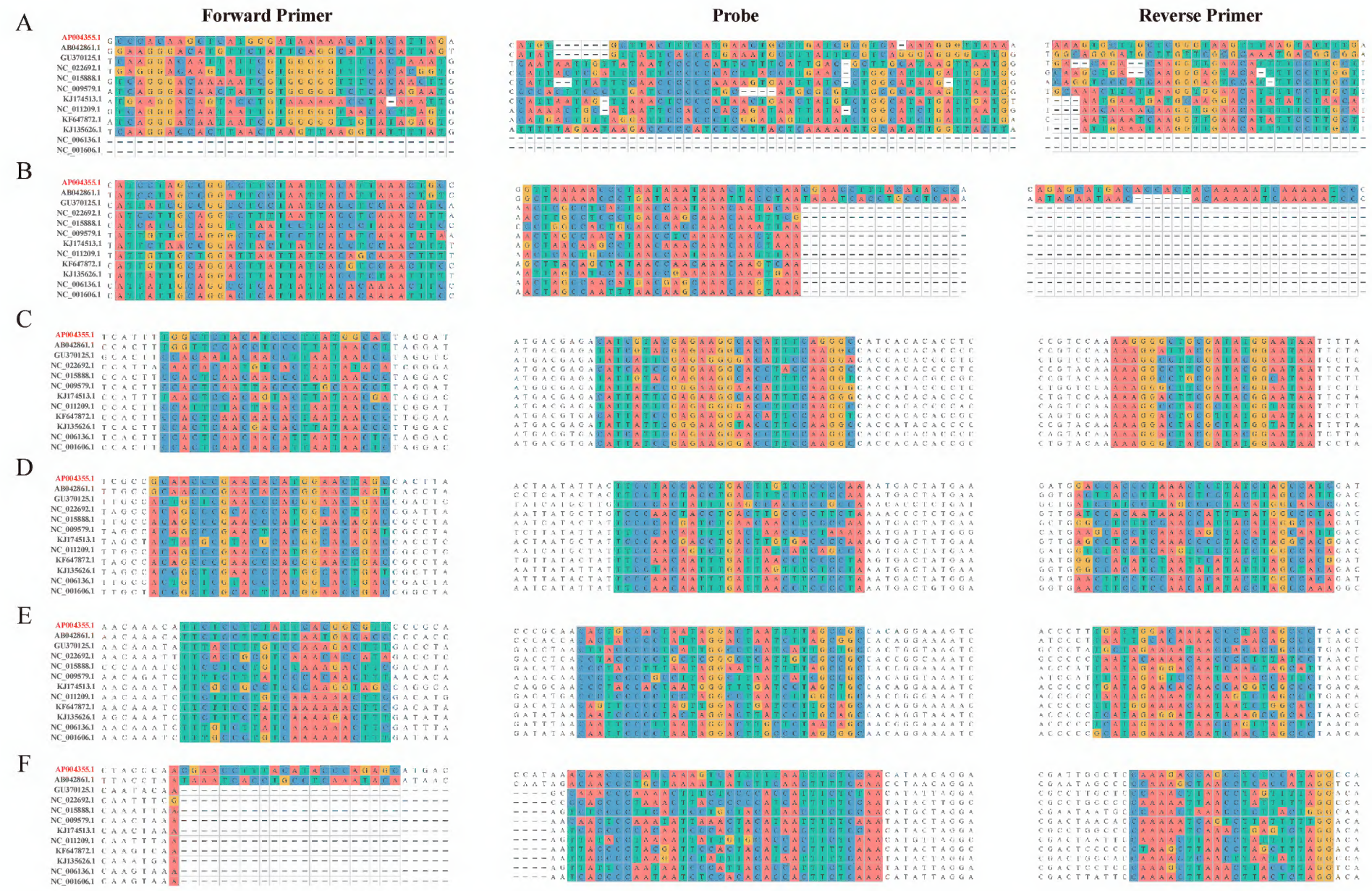

Figure 1. Sequence variation alignments of the RPA and qPCR primer/probe set in mitochondrion genome of the *Atractosteus spatula* and the 11 comparative species. **A.** RPAS1; **B.** RPAS2; **C.** qPCRS1; **D.** qPCRS2; **E.** qPCRS3; **F.** qPCRS4. The correspondence between sequences and names is as follows, AP004355.1: *A. spatula*; AB042861.1: *Lepisosteus oculatus*; GU370125.1: *Nile tilapia*; NC_022692.1: *Rhinogobius giurinus*; NC_015888.1: *Tachysurus fulvidraco*; NC_009579.1: *Coilia nasus*; KJ174513.1: *Acipenser sinensis*; NC_011209.1: *Misgurnus anguillicaudatus*; KF647872.1: *Hemiculter leucisculus*; KJ135626.1: *Pseudorasbora parva*; NC_006136.1: *Carassius auratus*; NC_001606.1: *Cyprinus carpio*.

and >85% interspecific divergence. RPA primers were designed with 30–35 bp length (Lobato and O'Sullivan 2018), avoiding homopolymer (>4 consecutive purines/pyrimidines) to prevent nonspecific hybridization. Furthermore, it has better incorporated terminal 3'-GC clamps (≥2 GC bp) to enhance polymerase binding stability during strand displacement (Piepenburg et al. 2006), with overall GC content maintained at 40–60%. Probes featured three essential modifications: a tetrahydrofuran (THF) site spacer, 5'-FAM and a 3'-C3 spacer. For qPCR, amplicon lengths were constrained to 100–150 bp for ensuring high amplification efficiency (Bustin et al. 2009). TaqMan probes were designed with 65–68 °C melting temperature (Tm) and 30–50% GC content, avoiding 5'-guanine residues to prevent fluorescence quenching. Primer-probe spatial arrangement maintained at least one bp spacing, with primers designed for 58–62 °C Tm (19–22 bp in length) using nearest-neighbor thermodynamics. For internal control of false negatives, a set of artificial primers and probe was also designed. Secondary structure formation of both primers and probes was minimized through in silico evaluation using mFold (Zuker 2003). Final primer/probe combinations were validated through in silico PCR against the NCBI Actinopterygii database (accessed 2023-12) using OBITools 1.01 (Boyer et al. 2016). The final sequences of the selected primers and probes of both assays are listed in Table 1. Primers and probes were synthesized by GENEWIZ Biotechnology Co., Ltd. (Suzhou, China).

Table 1. Primers/Probes for RPA-LFD and qPCR assay of *Atractosteus spatula*.

Name	Location	Sequences (5'-3')	
RPAS1	D-loop	Forward Primer	CCCACAAGCTCATGGGATAAAAACATACATTAG
		Reverse Primer	Biotin-CAAAATACTTAAACTTACCCGAGCAAGCACTTT
		Probe	FAM-ATGTGCTTACTTTCATGAACTGCTTGATCG/dSpacer/CGTCAAAAGGGTTAAA-C3-spacer
RPAS2	ND5	Forward Primer	ATCCTAGCCGGCCTTCTAATTACATTAAACTGC
		Reverse Primer	Biotin-GGATTTTTGATTTTTGTAGTGGTGTCATGCTCT
		Probe	FAM-TTAAAAACCCTAATAAATAAACTACCCAAC/dSpacer/GAACCTTTACATACCC-C3-spacer
qPCRS1	COX III	Forward Primer	GGCTCTACATCCCTTATGGCAC
		Reverse Primer	TTATTCCATATCGCAGCCCCT
		Probe	FAM-ATCGTACGAGAAGGCACATTTCAGGGC-BHQ1
qPCRS2	ND4L	Forward Primer	CAACCCGAACACATGGAACTAGC
		Reverse Primer	GATGGCTAGATAAGAGTTTAGGGTGGT
		Probe	JOE-TCCTACCACCTGACTTGTCTCCCCAA-BHQ1
qPCRS3	ND5	Forward Primer	TCTCCTCTATTCACGGCGTT
		Reverse Primer	GGGCTGTAGGGTTTTGTCCAATC
		Probe	Cy5-ACTGCCACTAATAGGACTAATTTTAGCCGC-BHQ1
qPCRS4	ND5	Forward Primer	CGAACCTTTACATACCCAGAGC
		Reverse Primer	CCTATGGAGAGGCTGGTCTTT
		Probe	FAM-CAACCCCATCAAAGTCATTTTTAATTTCTCCAA-BHQ1
qPCRIN		Forward Primer	CGGATCACTCTGAACTGGGA
		Reverse Primer	AGTTCCTCTAGCGTTGGACC
		Probe	ROX-TGGCCCAGCAAGAATCTATAACACGCATCA-BHQ1
		Template	CGGATCACTCTGAACTGGGATCTGCTGGCCCAGCAAGAATCTATAACACGCATCAGACGCGAACCACATGGTC-CAACGCTAGAGGAACT

Sample collections

Five live specimens (body length around 10 cm) of *A. spatula* provided by the Pearl River Fisheries Research Institute, Chinese Academy of Fishery Sciences, were maintained in aquarium tank (38 cm × 26 cm × 30 cm) with ddH_2O. For specificity detection, fin tissue samples were collected from *A. spatula* and closely related or cohabiting species, including *A. sinensis*, *C. auratus*, *C. nasus*, *C. carpio*, *H. leucisculus*, *L. oculatus*, *M. anguillicaudatus*, *N. tilapia*, *P. parva*, *R. giurinus* and *T. fulvidraco*. All procedures involving animals were conducted in accordance with the National Institutes of Health's Guide for the Care and the Ethical Standards of the Shanghai University (No. SHOUDW2024085). Genomic DNA of all species were extracted using the TIANamp Genomic DNA kit (Centrifugal Column Type, DP304-03). Besides, environmental DNA was filtered from two litters of aquarium water through a 0.45 μm mixed cellulose esters membrane (Pall Corporation, U.S.A.) with three replicates, and storage at cetyltrimethylammonium bromide (CTAB) preservation solution. To minimize the effect of humic acid, eDNA was extracted using the phenol-chloroform method and stored at -20 °C until next experiment. Both DNA concentrations were measured using a NanoDrop 3300 Fluorospectrometer (Thermo Fisher Scientific, Wilmington, DE, USA).

Assay optimization and in vitro specificity testing

To optimize the reaction conditions, genomic DNA (~20 ng/μL) was amplified under seven incubation temperatures (26, 29, 32, 35, 38, 41 and 44 °C) to determine the optimal reaction temperature. Reaction duration of 3, 6, 9, 12, 15, and

18 minutes were also evaluated at 38 °C to identify the optimal reaction time. The specificity of both RPA and qPCR primers was initially validated using genomic DNA from closely related or co-occurring species via conventional PCR, followed by gel electrophoresis to confirm the results. Furthermore, the specificity of the probes was further assessed using both RPA and qPCR. The RPA amplification was carried out in a 50 μL reaction volume using a commercial DNA isothermal rapid amplification kit (AMP-Future Biotech Co., Ltd., Changzhou, China), according to the manufacturer's protocol. Each RPA reaction mixture consisted of 29.4 μL of Buffer A, 2 μL each of 10 μM forward and reverse primers (final concentration 0.4 μM), 0.6 μL of 10 μM probe (final concentration 0.12 μM), 2 μL of DNA template (~20 ng/μL), 2.5 μL of Buffer B and another 11.5 μL ddH_2O. The mixture was vortexed with a Vortex-Genie 2 (Scientific Industries Co., Ltd, U.S.A.), centrifuged using a mini centrifuge (JOANLAB Equipment Co., Ltd, Zhejiang, China), and incubated in a dry bath incubator (Kylin-Bell Lab Instruments Co., Ltd, Jiangsu, China) at 38 °C for 15 minutes. Thereafter, each amplification product was diluted 1:20 with DNase/RNase-Free water, and 50 μL was applied to a gold nanoparticles-based dipstick (AMP-Future Biotechnology Co., Ltd., Shanghai, China) for visual detection. The qPCR was implemented with total volume of 25 μL mixture, containing 12.5 μL 2 × Superstart Premix (Probe qPCR) (Biori Biotech., Zhuhai, China), 0.5 μL each forward and reverse internal primer (10 μM), 0.5 μL internal probe (4 μM), 0.5 μL each forward and reverse specific primer (10 μM) and 0.5 μL specific probe (4 μM), 2 μL DNA template and 1 μL internal DNA template, remaining volume filled with ddH_2O. The mixture was heated at 95 °C for 5 minutes, followed by 45 cycles with denaturation at 95 °C for 20 seconds, annealing and extension at 60 °C for 30 seconds with Mic qPCR cycler (Liferiver Biotech., China). Meanwhile, cycle threshold (CT value) below 35 is considered detectable. To minimize the impact of exogenous sequences on the amplification system of the target fragment, exogenous DNA sequences should be kept to a minimum while ensuring detectable amplification. Thus, the optimal concentration of the internal control system was evaluated by testing a series of dilutions of primers (0.1~0.8 μM), probes (0.048~0.32 μM), and internal template (5×10^{-3} to 50 ng).

Evaluation and comparison of RPA and qPCR

To comparatively evaluate RPA and qPCR performance characteristics, a 10-fold serial dilution series was prepared with absolute copy numbers validated via ddPCR (Applied Biosystems QuantStudio Absolute Q Digital PCR System, Thermo Fisher Scientific) in accordance with MIQE guidelines (Huggett et al. 2013). Each dilution level underwent 10 technical replicates on both RPA and qPCR protocols. Because the outcome at each concentration is binary, probit regression was employed to model the relationship between DNA concentration and detection probability, as it is the standard method for estimating the LODs in qualitative molecular assays according to CLSI EP17-A2 protocols (MASS packages v7.3-65). Receiver operating characteristic (ROC) analysis was implemented in R (pROC package v1.18.4) using 2,000 stratified bootstrap replicates to estimate the area under the curve (AUC) with 95% confidence intervals.

On site validation of both assays

To simulate realistic aquatic environments, live specimen of *A. spatula* and sympatric fish species were maintained in aquaria filled with water collected from local freshwater ecosystems. In addition, an aquarium housing only *A. spatula* was established as the positive control, while natural water confirmed to be free of the target species served as the negative control, and DNase/RNase-Free water as the blank control. To further evaluate the practical performance of both assays, field validation was conducted at an *A. spatula* wild breeding pond at the Pearl River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Guangdong Province, China. Two litters of water sample were also filtered through 0.45 μm MCE membranes (Pall Corporation, U.S.A.) and the eDNA subsequently extracted using the Chelex 100 method (Gautam 2022). RPA-LFD and qPCR detection implemented as described in last sections.

Results

Specificity analysis of detection assays

The specificity of both RPA and qPCR assays was evaluated using genomic DNA (~30 ng/μL) from closely related and indigenous species, including *A. sinensis*, *A. spatula*, *C. auratus*, *C. nasus*, *C. carpio*, *H. leucisculus*, *M. anguillicaudatus*, *N. tilapia*, *P. parva*, *R. giurinus* and *T. fulvidraco*. Gel electrophoresis results demonstrated that both assays exclusively amplified the target DNA of *A. spatula*, with no cross-reactivity observed for any non-target species (Fig. 2). The RPA assay produced a clear positive signal only in samples containing *A. spatula* DNA. Similarly, qPCR amplification curves showed specific detection of the target species with no nonspecific amplification (Fig. 3).

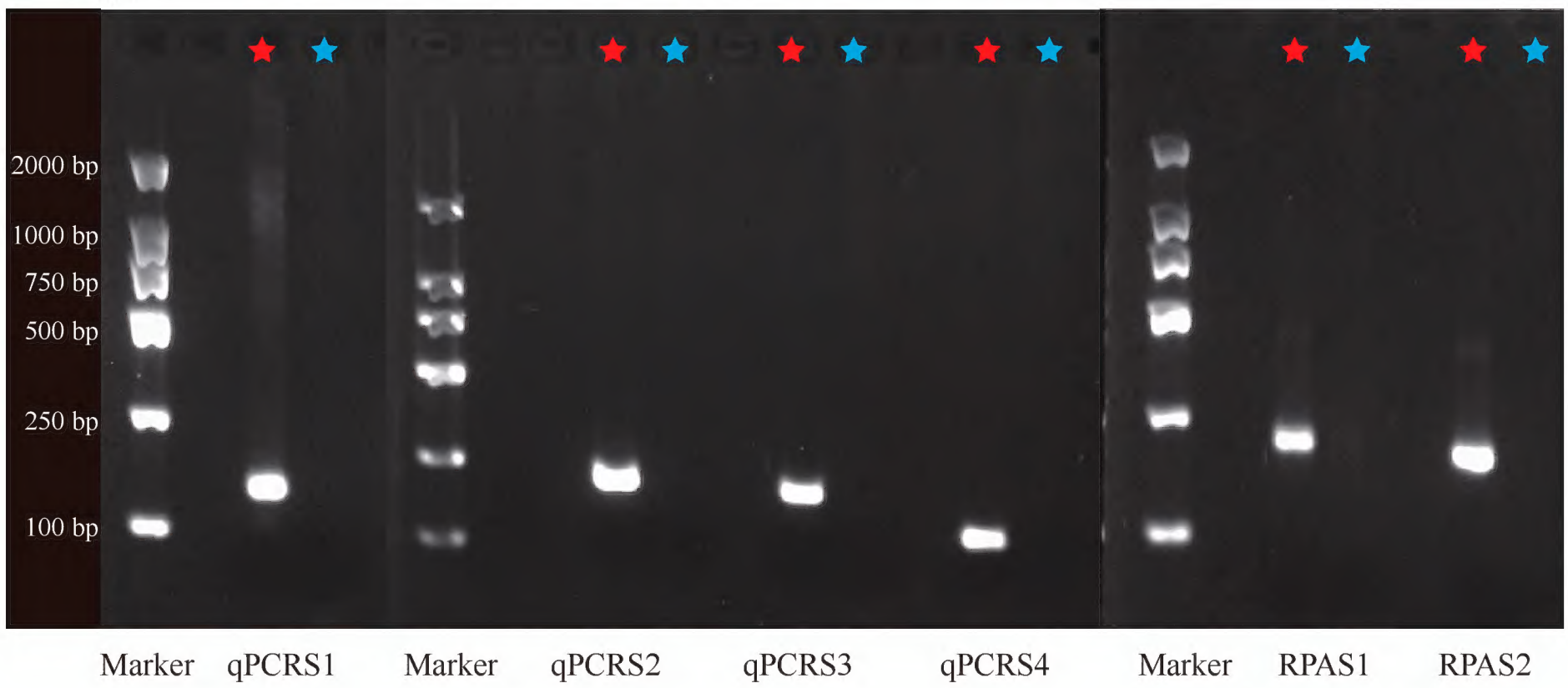

Figure 2. Gel electrophoresis pattern of primer specificity. The red star represents amplification of *A. spatula* genomic DNA, and the blue star represents amplification of DNA mixture from 11 background fishes.

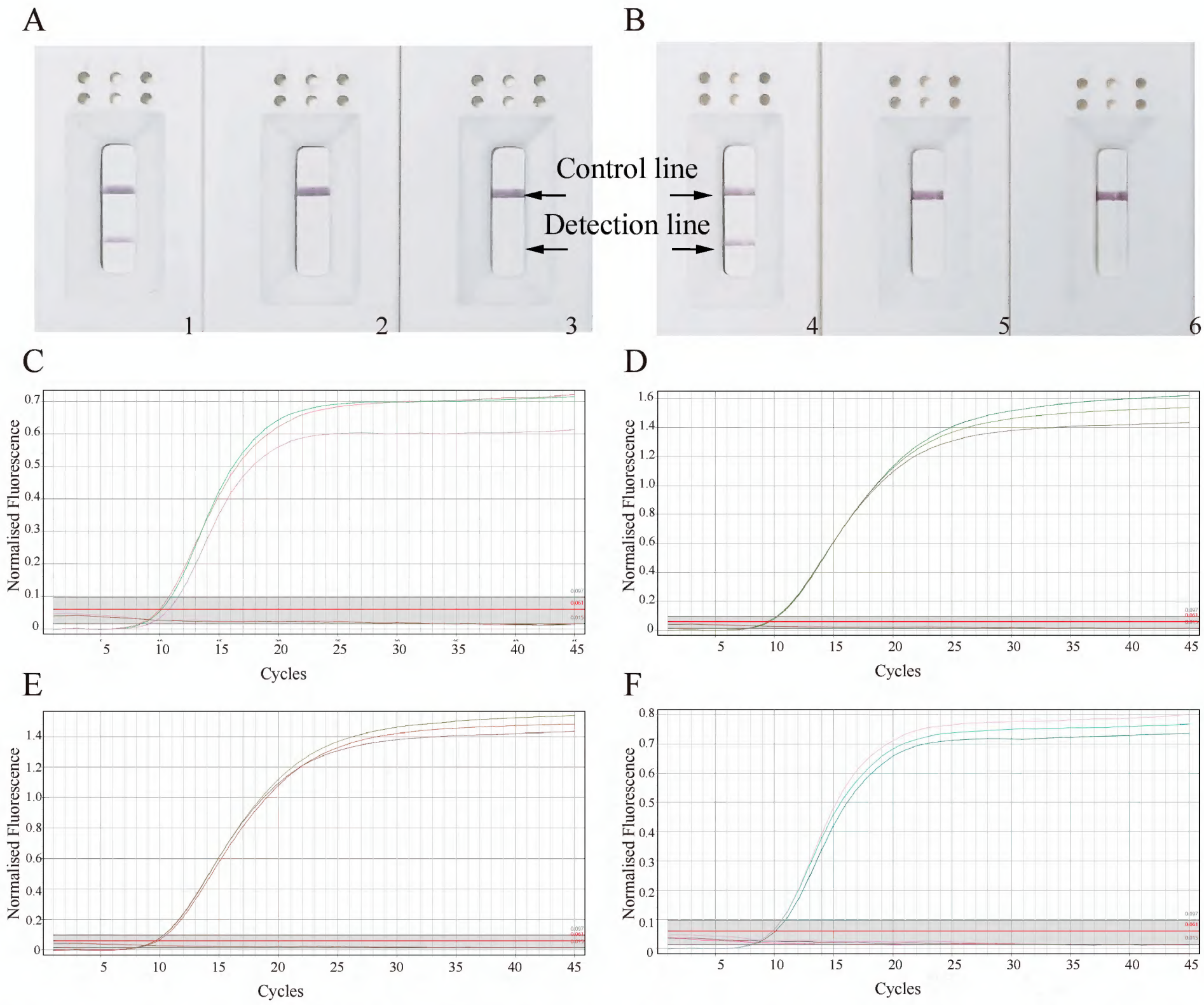

Figure 3. Assessment of the specificity of probes. RPAS1 (**A**) and RPAS2 (**B**), Lane 1, 4: Genomic DNA of *A. spatula*; Lanes 2, 5: mixed DNA from 11 non-target fish species; Lanes 3, 6: blank controls. Only genomic DNA being detected within qPCRS1 (**C**), qPCRS2 (**D**), qPCRS3 (**E**), qPCRS4 (**F**).

Optimization of two detection assays

To determine the most optimal reaction temperature for the RPA-LFD detection assay, the reaction mixture was incubated at intervals of 3 °C between 26 °C and 44 °C for 12 minutes using genomic DNA as the template. A positive signal was observed from 32 °C to 44 °C for the RPAS1 set, and from 32 °C to 41 °C for RPAS2 (Fig. 4A). Based on consistent performance across all primer–probe sets, 38 °C was selected as the optimal reaction temperature. Subsequently, reaction duration ranging from 3 to 18 minutes were tested at this temperature. As shown in Fig. 4B, a faint positive signal was first detectable after 9 minutes, with signal intensity increasing noticeably by 12 minutes. To ensure reliable detection even under low concentrations within the minimal possible time frame, a 15-minute reaction time was chosen as optimal.

To reduce the risk of false-negative results in qPCR, an exogenous synthetic sequence was introduced as an internal control system, which included corresponding primers, probes, and template (refer to qPCRIN in Table 1). The qPCRIN was added only at the PCR stage to verify the reaction competence. To establish this system, the minimal amount of exogenous DNA required for reliable amplification

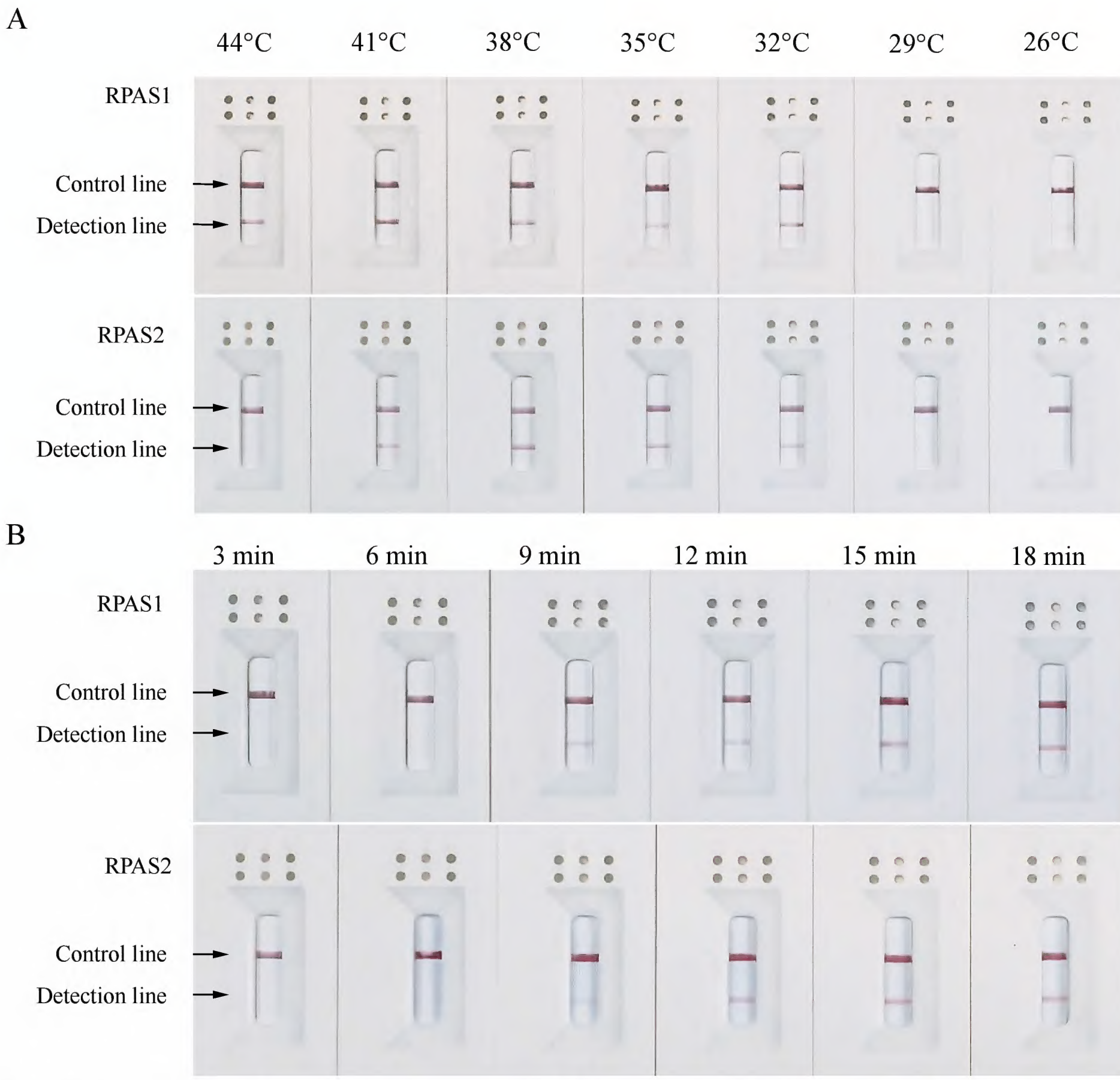

Figure 4. Optimization of the RPA-LFD assay for three sets of primers and probes. **A.** Optimization of the RPA reaction temperature, positive signal manifested from 32 °C to 44 °C of RPAS1, and from 32 °C to 41 °C for RPAS2; **B.** Optimization of the RPA reaction time, positive signal gradually appeared after 9 minutes and became stable at 12 minutes.

was determined through a series of controlled experiments. As shown in Fig. 5A, the internal DNA template (0.005 ng/μL) demonstrated the capacity to achieve effective amplification, exhibiting a mean CT value of 18. Meanwhile, CT values remained consistent across a range of primer and probe concentrations (Fig. 5B, C), indicating stable amplification performance. Consequently, an internal quality control system was established using a DNA template of 0.005 ng/μL, a primer concentration of 0.2 μM and a probe concentration of 0.16 μM.

Sensitivity and comparison of detection assays

The sensitivity of both assays was assessed using a serial dilution *A. spatula* genomic DNA, ranging from 0.68 copies/μL to 434.00 copies/μL. The reliability of each detection method was further evaluated using receiver operating characteristic

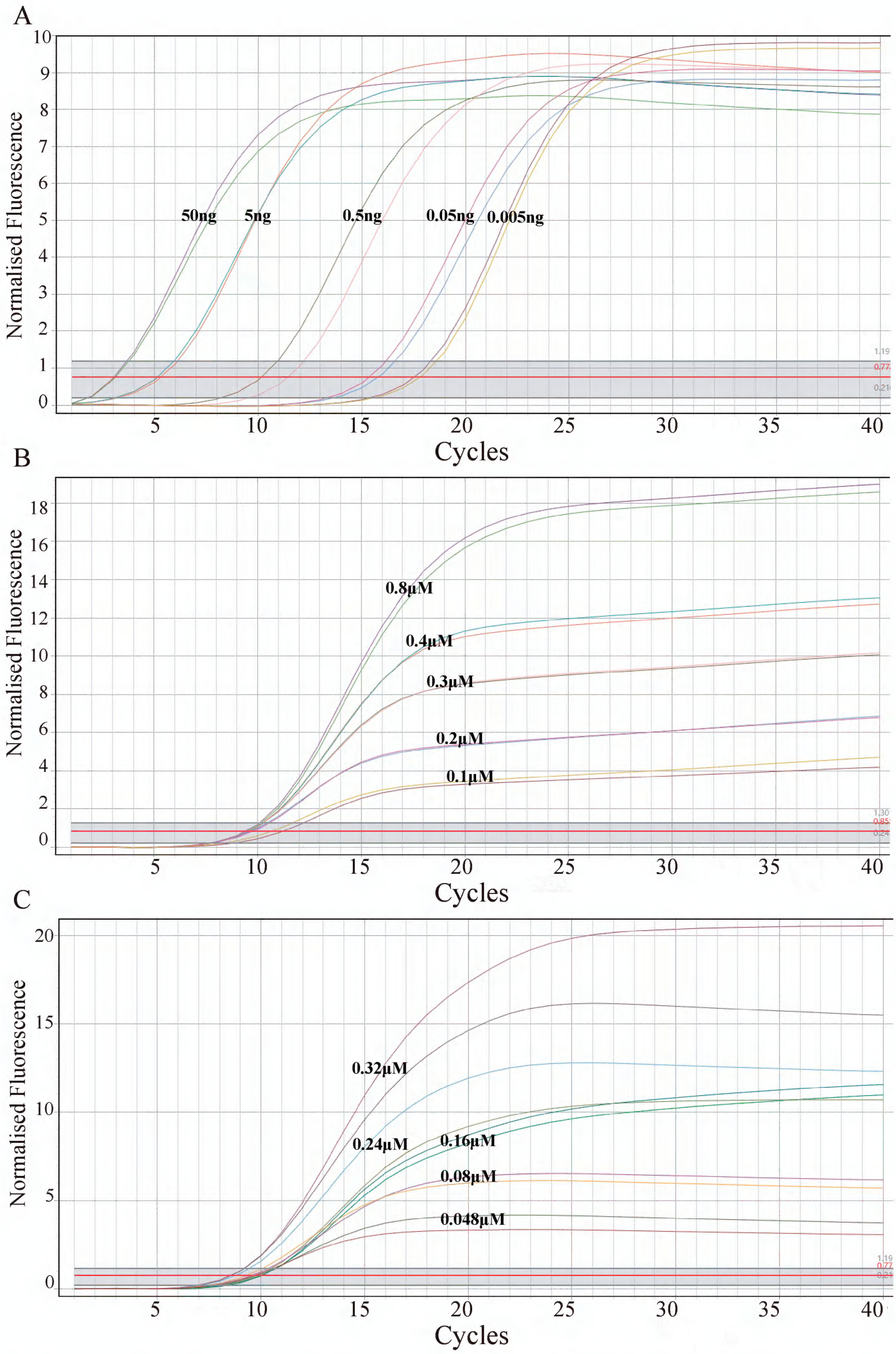

Figure 5. Examination of appropriate concentration in internal quality control system for qPCR. **A.** Amount of DNA template added in qPCR mixture; Concentration of primer (**B**) and probe (**C**) added in qPCR mixture.

(ROC) curve analysis. For RPA-LFD assay, the first primer-probe set RPAS1 achieved 100% detection rate (10/10) at a concentration of 15.88 copies/μL, but only 50% (5/10) at 2.83 copies/μL. Its 95% limit of detection (LOD) was determined to be 4.21 copies/μL (95% CI: 3.49~5.08 copies/μL) by probit regression (Figs 6A, 7). The RPAS2 set showed reduced sensitivity, with 90% detection (9/10) at 80.85 copies/μL and only faint, infrequent signals (6/10) at 15.88 copies/μL. Its 95% LOD was 84.17 copies/μL (95% CI: 46.50~152.38 copies/μL; Figs 6B, 7).

The qPCR detection assay achieved higher overall sensitivity compared to the RPA-LFD detection method. All qPCR primer-probe sets except qPCRS3 achieved 100% detection at 15.88 copies/μL; qPCRS3 reached 90% (9/10) at this concentration. With a concentration of 2.83 copies/μL, detection rates were as follows: qPCRS1 (90%, 9/10), qPCRS2 (60%, 6/10), qPCRS4 (50%, 5/10), and qPCRS3 (10%, 1/10). Probit regression ranked the sensitivity of the qPCR sets as follows: qPCRS1 was most sensitive (LOD = 4.12 copies/μL; 95% CI: 0.67~25.27 copies/μL), followed by qPCRS4 (10.6 copies/μL; 95% CI: 6.15~18.25), qPCRS2 (11.39 copies/μL; 95% CI: 5.76~22.52 copies/μL), and qPCRS3 (20.18 copies/μL; 95% CI: 12.23~33.32 copies/μL; Fig. 7).

The area under the ROC curve (AUC) was used as the primary metric for diagnostic performance. The qPCRS1 set yielded the highest AUC (0.936), followed by RPAS1 (0.894). Both qPCRS2 and RPAS2 shared an AUC of 0.864, while qPCRS4 and qPCRS3 scored 0.784 and 0.600, respectively (Fig. 7).

Complete on-field validations using RPA and qPCR

To evaluate assay performance under realistic conditions, environmental DNA (eDNA) samples were collected from four sources: (1) a positive control tank containing *A. spatula* in natural freshwater; (2) a natural lake confirmed free of the

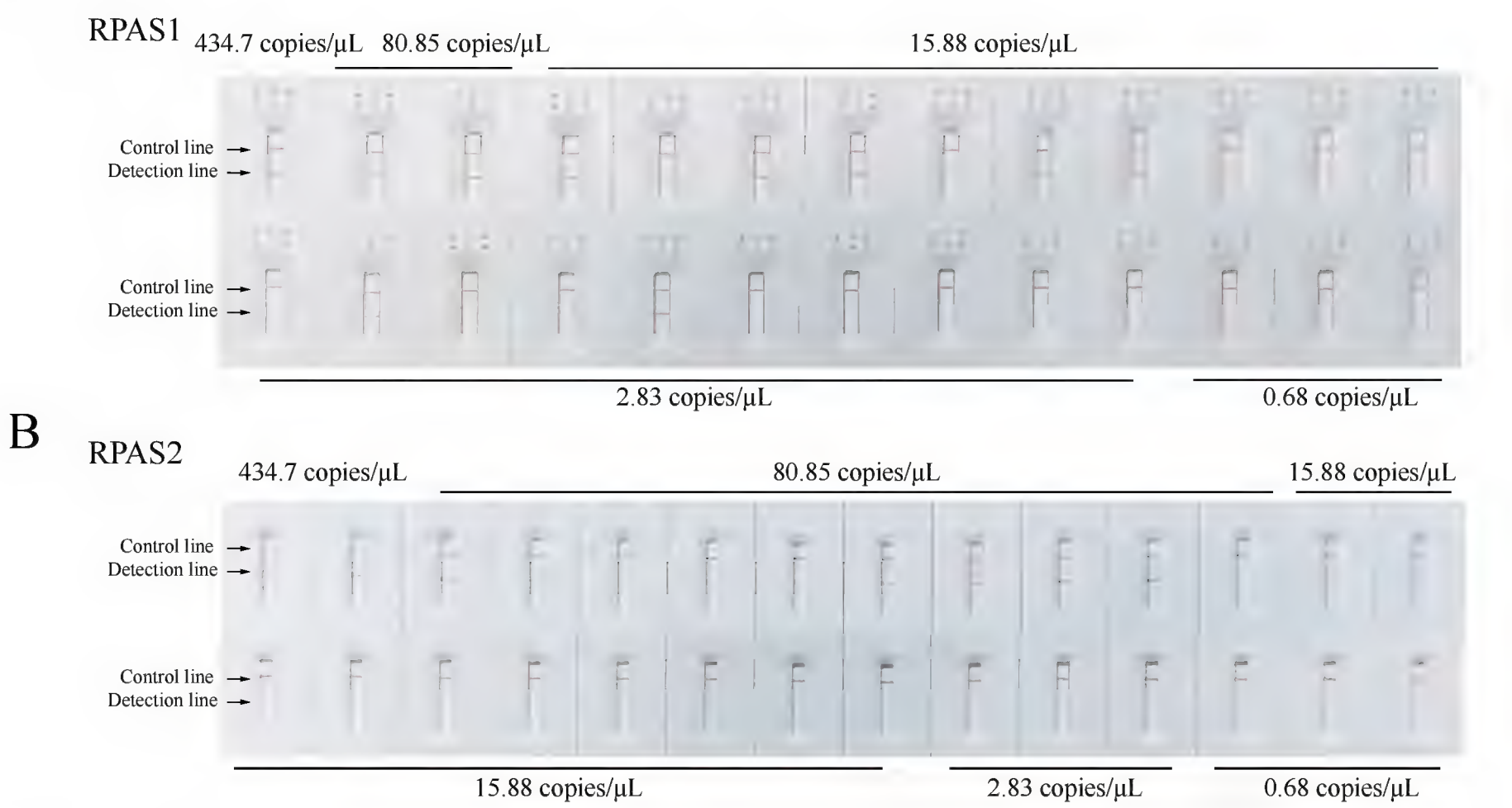

Figure 6. Assessment of the sensitivity of primer-probe pairs of RPA-LFD. **A.** For RPAS1; **B.** For RPAS2. Each sample was detected with ten technical replicates at different concentration.

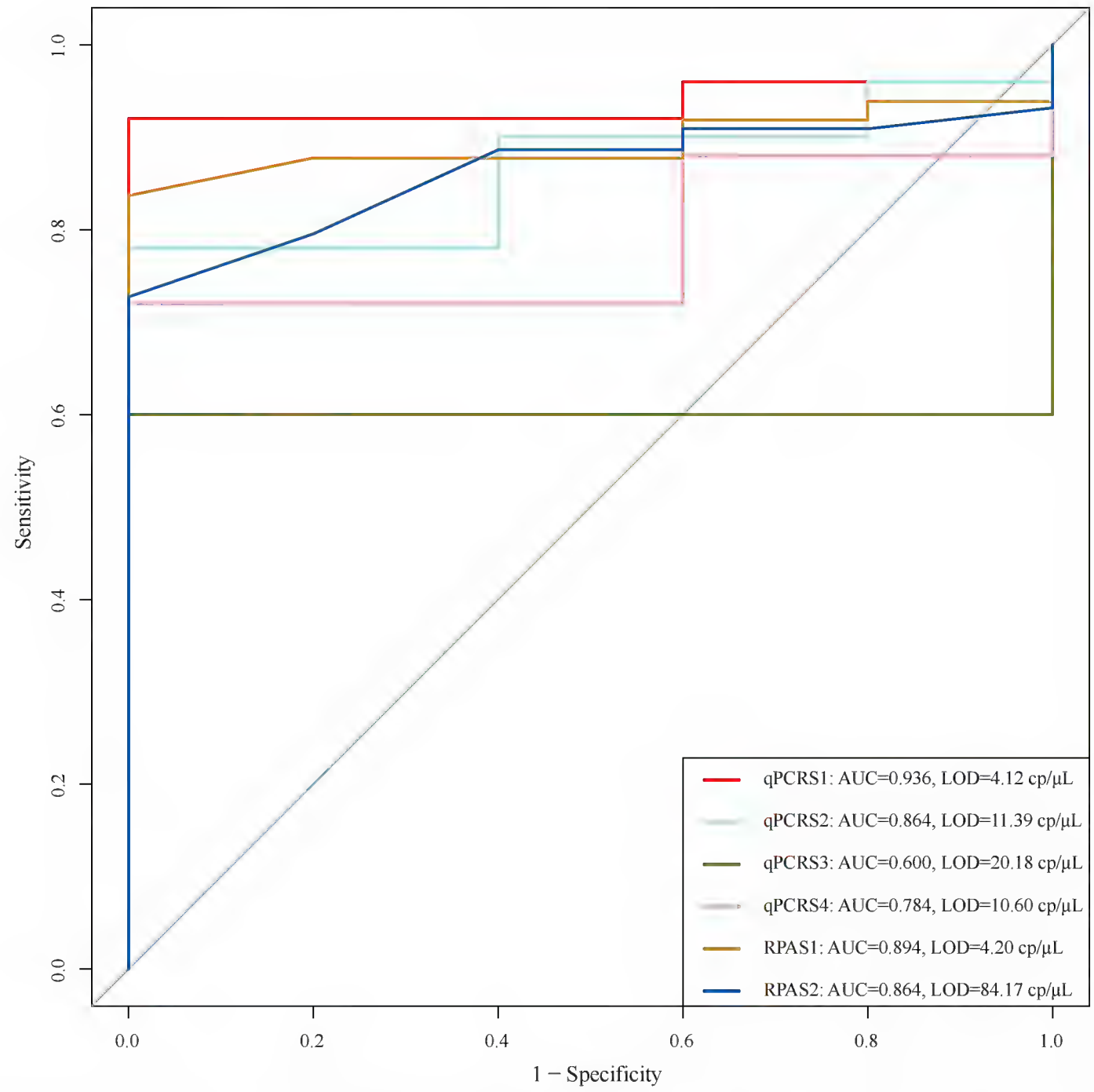

Figure 7. Receiver operating characteristic (ROC) curve of RPA and qPCR detection assays. AUC represents the area under the ROC curve, the AUC range from 0.5 to 1, when AUC is more than 0.9 indicated a relatively high accuracy, with AUC more than 0.7 refers the detection method performs well. LOD refers to the minimum concentration of the target analyte that can be detected from the sample by a specific analytical method at a confidence level of 95%.

target species as a negative control; (3) a wild outdoor breeding pond of *A. spatula* for field validation; and (4) DNase/RNase-Free water as a blank control. In order to shorten the duration, eDNA was extracted using Chelex100, and reached a concentration of 4.5 ng/μL (as measured in laboratory). Positive signals were observed with DNA template from positive aquarium and wild outdoor breeding pool for both RPAS1 and RPAS2 (Figs 8A, 7B), with no positive signal was observed with DNA from negative control. Further validation was performed using qPCR assays, with all primer-probe pairs demonstrated effective detection of *A. spatula* positive control eDNA sample and only qPCRS3 didn't pass the field test (Fig. 8C–F).

Discussion

Biological invasions, fueled by globalization and human population expansion, are among the most pressing environmental challenges of the 21st century (Pyšek et al. 2020; Peller and Altermatt 2024), causing biodiversity decline and ecosystem degradation through resource competition and functional disruption (Pejchar and Mooney 2009; Colberg et al. 2024). In this study, we developed and rigorously validated a novel RPA-LFD assay for the rapid, field-deployable detection of the invasive alligator gar (*Atractosteus spatula*), achieving a sensitivity of 4 copies/μL within 15 minutes

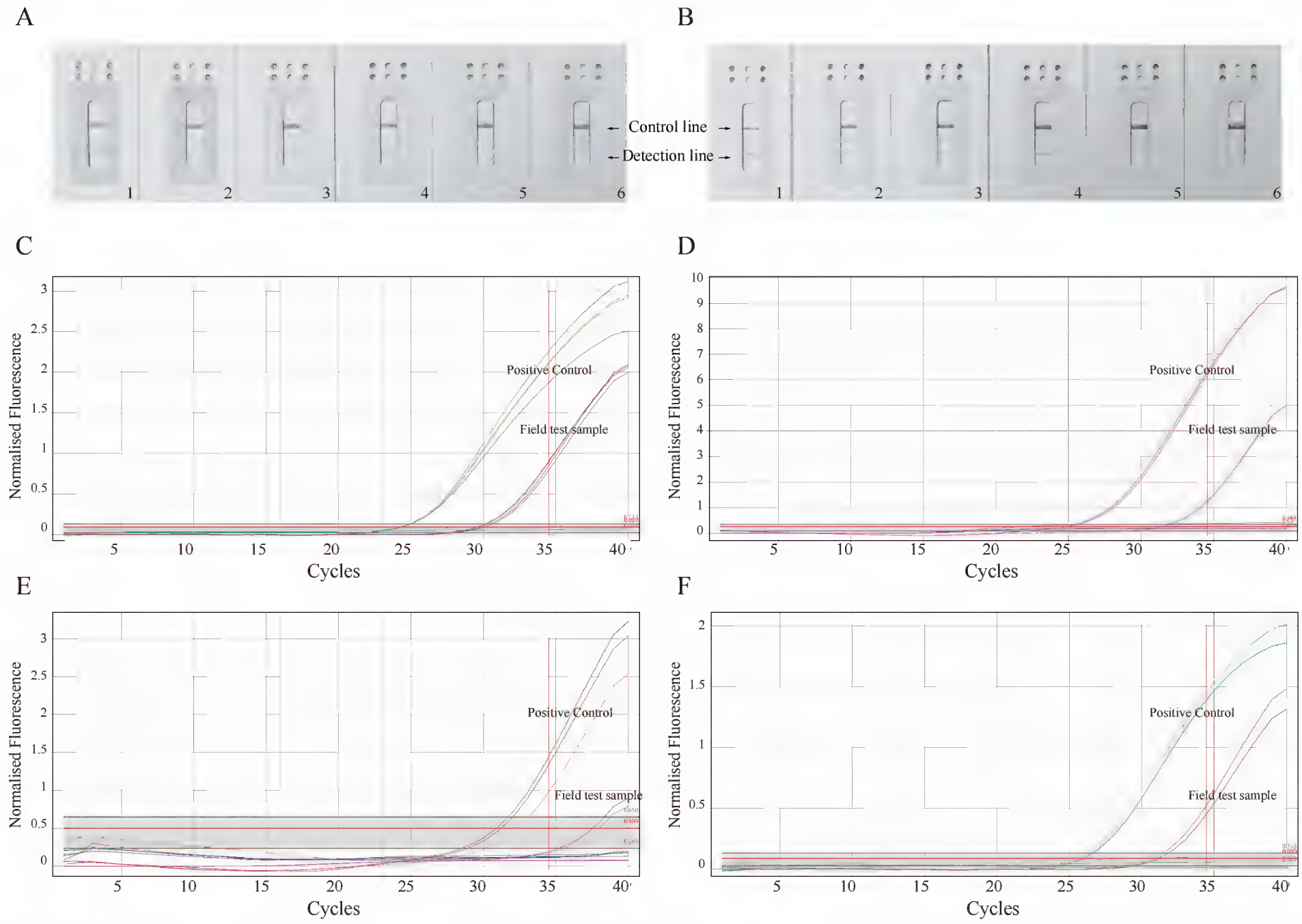

Figure 8. On field test of RPA and qPCR assay. RPAS1 (**A**) and RPAS2 (**B**): Lane1: positive control; Lanes2-4: three technical replicates of *A. spatula* field water eDNA; Lane 5: eDNA from semi-closed lake as negative control; Lane 6: blank control. For qPCR detection assay, except qPCRS3 (**E**), qPCRS1 (**C**), qPCRS2 (**D**) and qPCRS4 (**F**) achieved a 100% detection rate with all four technical replicates being detected.

under isothermal conditions, which perform comparable to the qPCR gold standard. By providing an equipment independent molecular tool that enables early warning surveillance in resource-limited settings, this work advances proactive conservation strategies and democratizes access to invasive species monitoring, with direct implications for safeguarding freshwater biodiversity (Yang et al. 2024; Leugger et al. 2025).

Mitochondrial DNA (mtDNA) is widely used in eDNA assays due to its high copy number, resistance to degradation, and utility in vertebrate species identification (Freeland 2016; Furlan et al. 2020; Doorenspleet et al. 2025), with commonly used markers include the cytochrome b (*Cyt b*) gene (Keskin et al. 2016; Sakai et al. 2019), cytochrome *c* oxidase subunit I (*COI*) gene (Uchida et al. 2020; Holman et al. 2022), and the D-loop region (Yoshitake et al. 2019). In this study, we screened the entire mitogenome of *A. spatula* for potential markers and identified specific regions within the *D-loop*, NADH dehydrogenase 5 (*ND5*), NADH dehydrogenase 4L (*ND4L*), and cytochrome *c* oxidase subunit III (*COXIII*) genes suitable for assay design. The integration of eDNA with RPA-LFD has enabled reliable field detection of species such as *Prorocentrum minimum* (Fu et al. 2020) and the Chinese Giant Salamander (*Andrias davidianus*) (Ling et al. 2024). Similarly, eDNA-based qPCR is well-established for sensitive and specific AIS monitoring (Klymus et al. 2015; Andres et al. 2021). In fact, the choice between detection methods often involves a trade-off between marginal gains in analytical sensitivity and substantial

gains in operational practicality in field biology applications. In our study, qPCR, as the gold-standard method, exhibited higher analytical sensitivity than RPA-LFD, with a lower limit of detection (LOD < 25 copies/μL). Although one qPCR assay (qPCRS3) showed reduced performance during field validation, which is consistent with its lower AUC, the best-performing RPA assay (RPAS1) demonstrated efficiency comparable to qPCRS1, with LOD values around 4 copies/μL and AUC above 0.89 with eliminating the need for thermal cycling equipment. Recently, the CRISPR-Cas systems have emerged as a revolutionary genome-editing tool, enabling significant advances in nucleic acid detection. The RNA-guided nuclease Cas12a, in particular, is widely used for gene editing and molecular diagnostics (Kellner et al. 2019; Baerwald et al. 2020). CRISPR-based assays have recently been developed for aquatic species monitoring, such as *Culter erythropterus* and the invasive species *A. spatula* (Wei et al. 2023, 2025; Zhang et al. 2024). The sensitivity of CRISPR/Cas 12 based detection for *A. spatula* was 10.3 fM within 50 min, and the RPA-CRISPR/Cas12a technique can detect 6.0 copies per micro-litter of fish eDNA within 2 hours according to Wei et al. (2025). By comparison, our RPA-LFD assay achieved a shorter detection time (15 minutes) and higher sensitivity, offering a substantial advantage for field applications requiring rapid on-site decision-making. Despite its promising performance, the RPA-LFD assay developed herein is inherently qualitative or semi-quantitative and therefore cannot provide precise DNA copy number estimates. Furthermore, although the assay was successfully validated in a natural breeding pond, its performance in more complex water matrices (e.g., those with high humic acid content, elevated turbidity, or eutrophic conditions) remains to be evaluated, as potential inhibitory substances may affect amplification efficiency (Mauvisseau et al. 2022). Further testing across a wider range of environmental conditions and water chemistry profiles will be necessary to confirm the robustness and broad applicability of these two methods.

Citizen science initiatives play an increasingly vital role in invasive species surveillance by enabling the collection of large-scale, long-term datasets (Pocock et al. 2024). The rapid on-site detection method for *A. spatula* developed in this study holds strong potential for extension to artificial water bodies, such as urban ponds, reservoirs, and ornamental lakes in residential and park settings, where early detection is often most urgently needed (Truelove et al. 2019). The adoption of user-friendly molecular tools, such as the RPA-LFD and Chelex-100 extraction methods implemented here, can facilitate public participation by simplifying sample processing and reducing time and cost barriers (Larson et al. 2020; Pocock et al. 2024). These approaches enable structured, scientifically valid monitoring while expanding early detection capacity. Thus, this study provides not only a novel detection method but also a distributed monitoring framework that integrates modern molecular technology with public engagement. It is expected to shift invasive species monitoring from a passive and reactive approach to an active and predictive strategy, thereby offering critical technological support for enhancing national aquatic ecological security.

Conclusions

In conclusion, we successfully developed robust and field-deployable RPA-LFD and qPCR assays for the specific detection of *Atractosteus spatula*, a key invasive species in China. The assays exhibited high sensitivity, with detection limits as low

as 4 copies/μL, and strong specificity without cross-reactivity to non-target species. Field validation conducted in a natural breeding pond confirmed the effectiveness of both methods, with the RPA-LFD assay showing particular promise for citizen science initiatives due to its simplicity and minimal equipment requirements. This study provides valuable molecular resources for the proactive surveillance of *A. spatula* and underscores the potential of combining modern genetic tools with environmental DNA analysis for combating biological invasions in aquatic ecosystems.

Additional information

Conflict of interest

The authors have filed two patent applications related to the content of this study (Application No. 1: CN202311727119.4; Application No. 2: CN202311714669.2). These patents may represent a potential conflict of interest.

Ethical statement

All procedures involving animals were conducted in accordance with the National Institutes of Health's Guide for the Care and the Ethical Standards of the Shanghai Ocean University (No. SHOUDW2024085).

Use of AI

No use of AI was reported.

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Author contributions

Hui Wang, Shuyi Li, Xiaolong Lin and Chenhong Li conceived the ideas and designed methodology; Hui Wang, Shuyi Li conducted laboratory work; Xidong Mu supplied on-field test site; Shuyi Li and Xidong Mu implemented data analysis; Hui Wang made further data analysis; Hui Wang and Shuyi Li drafting the article, Xiaolong Lin, Chenhong Li and Xidong Mu led the revising of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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Data availability

The authors confirm that the data supporting the findings of this study are available at https://figshare.com/s/f33619806e08e2ad4ff9. All mitochondrion genome sequences could be obtained from NCBI through accession number listed in article.

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